Available online at www.sciencedirect.com
ScienceDirect
International Journal for Parasitology 38 (2008) 561–569
www.elsevier.com/locate/ijpara

# Population structure and mouse-virulence of *Toxoplasma gondii* in Brazil

H.F.J. Pena [a], S.M. Gennari [a], J.P. Dubey [b], C. Su [c,*]

[a] *Department of Preventive Veterinary Medicine and Animal Health, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Av. Prof. Dr. Orlando Marques de Paiva, 87, CEP 05508-270, São Paulo, Brazil*
[b] *United States Department of Agriculture, Agricultural Research Service, Animal and Natural Resources Institute, Animal Parasitic Diseases Laboratory, Building 1001, Beltsville, MD 20705-2350, USA*
[c] *Department of Microbiology, The University of Tennessee, Knoxville, TN 37996-0845, USA*

Received 25 May 2007; received in revised form 3 September 2007; accepted 6 September 2007

**Abstract**

Recent studies found that isolates of *Toxoplasma gondii* from Brazil were biologically and genetically different from those in North America and Europe. However, to date only a small number of isolates have been analysed from different animal hosts in Brazil. In the present study DNA samples of 46 *T. gondii* isolates from cats in 11 counties in São Paulo state, Brazil were genetically characterised using 10 PCR restriction fragment length polymorphism markers including *SAG1*, *SAG2*, *SAG3*, *BTUB*, *GRA6*, c22-8, c29-2, L358, *PK1* and Apico. An additional marker, CS3, that locates on chromosome VIIa and has previously been shown to be linked to acute virulence of *T. gondii* was also used to determine its association to virulence in mice. Genotyping of these 46 isolates revealed a high genetic diversity with 20 genotypes but no clonal Type I, II or III lineage was found. Two of the 46 isolates showed mixed infections. Combining genotyping data in this study with recent reported results from chickens, dogs and cats in Brazil (total 125 isolates) identified 48 genotypes and 26 of these genotypes had single isolates. Four of the 48 genotypes with multiple isolates identified from different hosts and locations are considered the common clonal lineages in Brazil. These lineages are designated as Types BrI, BrII, BrIII and BrIV. These results indicate that the *T. gondii* population in Brazil is highly diverse with a few successful clonal lineages expanded into wide geographical areas. In contrast to North America and Europe, where the Type II clonal lineage is overwhelmingly predominant, no Type II strain was identified from the 125 Brazil isolates. Analysis of mortality rates in infected mice indicates that Type BrI is highly virulent, Type BrIII is non-virulent, whilst Type BrII and BrIV lineages are intermediately virulent. In addition, allele types at the CS3 locus are strongly linked to mouse-virulence of the parasite. Thus, *T. gondii* has an epidemic population structure in Brazil and the major lineages have different biological traits.


*Keywords:* *Toxoplasma gondii*; PCR-RFLP; Genetic diversity; Virulence; Cats; Chicken; Dog; Brazil

## 1. Introduction

*Toxoplasma gondii* is a zoonotic protozoan parasite that can infect all warm-blooded animals. It is estimated that one-third of the global human population is chronically infected with this parasite (Dubey and Beattie, 1988; Tenter et al., 2000). Humans can become infected by ingesting tissue cysts from undercooked meat or consuming food or drink contaminated with oocysts shed in the faeces of infected cats (Dubey, 2004). In Brazil, 50–80% of the adult population has antibodies to *T. gondii* (Bahia-Oliveira et al., 2003). Water has been identified as the major source of *T. gondii* transmission to humans in endemic areas (Bahia-Oliveira et al., 2003; De Moura et al., 2006).

In Brazil, *T. gondii* is an important protozoan parasite involved in human diseases. It causes most cases of cerebral focal lesions and is the third most common pathogen related to complications in acquired immunodeficiency

* Corresponding author. Tel.: +1 865 974 4015; fax: +1 865 974 4007.
*E-mail address:* csu1@utk.edu (C. Su).

0020-7519/$34.00 
doi:10.1016/j.ijpara.2007.09.004

syndrome (AIDS) patients (Colombo et al., 2005). It also causes 30–50% of all cases of posterior uveitis in both immunocompetent and immunocompromised individuals, with a high frequency of ocular toxoplasmosis reported in Erechim County, Southern Brazil (Glasner et al., 1992). PCR restriction fragment length polymorphism (PCR-RFLP) genotyping of the SAG2 locus suggested that Type I related strains were responsible for the ocular infections in this population (Vallochi et al., 2005). Recently, samples from human patients in Brazil with ocular toxoplasmosis were studied by multilocus RFLP and intron sequence analysis. The results revealed several non-typical haplotypes (Khan et al., 2006). All these studies indicate that *T. gondii* is of importance to human health in Brazil.

*Toxoplasma gondii* isolates of human and animals from Europe and North America have been classified into one of three genetic lineages (Types I, II, III) based on multilocus enzymes electrophoresis, PCR-RFLP and microsatellite typing (Dardé et al., 1992; Howe and Sibley, 1995; Ajzenberg et al., 2002). The parasite was previously considered clonal with very low genetic diversity. However, recent studies on isolates from animals or humans from remote geographical regions revealed higher genetic variability than previously reported (Ajzenberg et al., 2004; Lehmann et al., 2004). It was shown that isolates of *T. gondii* from Brazil are genetically different from those in North America and Europe (Lehmann et al., 2004, 2006; Ferreira et al., 2006; Khan et al., 2006; Su et al., 2006; Dubey et al., 2007a,b).

In this study, we genotyped 46 *T. gondii* isolates from cats in São Paulo state, Brazil, by multilocus PCR-RFLP markers. A new marker, CS3, which locates on chromosome VIIa and was previously shown to be linked with acute virulence of *T. gondii*, was also used to determine its association to parasite virulence in mice. The genotyping data obtained in this study was combined with recently published typing results from isolates of chickens (Dubey et al., 2007b), cats (Dubey et al., 2004; Su et al., 2006), and dogs (Dubey et al., 2007a) to reveal the population structure of the parasite in Brazil. The results showed that *T. gondii* has an epidemic population structure with a few expanded clonal lineages. This is in contrast to the population structure in North America and Europe, in which three clonal lineages (Types I, II and III) predominate and account for over 95% of the isolates (Howe and Sibley, 1995). The mouse-virulence of parasite isolates in Brazil is associated with the genotypes, and the allele types at marker CS3 are strongly linked to mouse-virulence.

## 2. Materials and methods

### 2.1. Toxoplasma gondii isolates

A total of 46 *T. gondii* cat isolates from 11 counties in São Paulo state, Brazil (Pena et al., 2006) were included in this study for genotyping. The counties were located 20–500 km apart from each other. The isolates were obtained by bioassay in mice. Fifteen reference strains of *T. gondii* were also included in genotyping. These included Type I strains (RH, GT1, VEL, ENT), Type II strains (PTG, PE, PIH), Type III strains (CTG, VEG, STRL) and other strains (Cougar, CAST, GPHT, MAS, P89).

### 2.2. Multilocus PCR-RFLP genotyping

*Toxoplasma gondii* DNA was extracted from tissues of two infected mice from each group. Strain typing was performed using genetic markers *SAG1*, *SAG2, SAG3*, *BTUB*, *GRA6*, c22-8, c29-2, L358, *PK1* and Apico as described previously (Su et al., 2006; Dubey et al., 2007b). An additional marker named CS3 that is located on *T. gondii* chromosome VIIa was included in the present study. This marker was included because it has been proved to be linked with acute virulence of the clonal type I *T. gondii* strains (Khan et al., 2005). For genotyping of CS3, the target DNA sequence was amplified by nested PCR. The external primers (794 bp products) are: CS3-Fext, GTGTATCTCCGAGGGGGTCT; CS3-Rext, TGTGAC TTCTTCGCATCGAC; and the internal primers (557 bp products) are: CS3-F, AGCGGATTTCCAACACTGTC; CS3-R, CTGCTGCATTCACAAACTCC. The target DNA sequences were first amplified by multiplex PCR using external primers for all markers, followed by nested PCR for individual markers for genotyping as described previously (Su et al., 2006; Dubey et al., 2007b). For the marker CS3, PCR products were digested by restriction enzymes N1aIII and MboI at 37 °C for 60 min and the DNA banding pattern revealed in 2.5% agarose gel.

### 2.3. Data analysis

Genotyping results for the 46 *T. gondii* cat isolates from 11 counties in São Paulo state were combined with recently reported results from chickens, dogs and cats in Brazil to form a composite dataset of 125 isolates (Dubey et al., 2004, 2007a,b; Pena et al., 2006; Su et al., 2006). To reveal the phylogenetic relationship of all parasite isolates, the composite dataset of multilocus PCR-RFLP genotyping was analysed by SplitsTree4 (Huson, 1998; Huson and Bryant, 2006). The result is presented as a reticulated network, which is preferred to the traditional bifurcating phylogenetic tree in describing complex relationships in population biology (Morrison, 2005).

To determine if there is an association between multilocus genotypes and virulence phenotypes in mice, data of virulence in mice from the composite dataset was retrieved from previously published reports (Dubey et al., 2004, 2007a,b; Pena et al., 2006; Su et al., 2006). In these studies, up to five outbred mice were infected s.c. with 1 ml of animal tissue preparation that contained an unknown number of *T. gondii*. Here, parasite virulence is defined based on the mortality of positively infected mice within 4 weeks of infection and it is categorised into three groups including virulent, intermediately virulent and non-virulent. Virulent

is defined as 100% mortality of infected outbred mice within 4 weeks, intermediately virulent is greater than 30% but less than 100% mortality of infected mice, and non-virulent is less than or equal to 30% mortality.

To determine if the CS3 marker can be used to predict parasite virulence in mice, the $\chi^2$ test or the Fisher exact test were used to compare associations between CS3 allele types of the cat isolates and the number of mice that died within 4 weeks of infection (Pena et al., 2006). $P$ values $\leqslant 0.05$ were considered significant.

## 3. Results

### 3.1. Multilocus PCR-RFLP genotyping of 46 cat isolates from São Paulo

Genotyping results of the 46 *T. gondii* cat isolates at all 11 markers are summarised in Table 1. Twenty genotypes (#1 to #20) were revealed for these Brazilian cat isolates. Of the 20 genotypes identified, seven (#1 to #7) are characterised with two or more isolates, whilst 13 genotypes (#8 to #20) have a single isolate. No clonal Types I, II or III lineages were found. Mixed infections were found in two isolates (TgCat43 and 49) from one county and the complex mixture of two alleles was observed at five loci: *SAG1* (I and u-1), c22-8 (I and II), c29-2 (I and III), *PK1* (II and u-1) and CS3 (I and II).

The genotype #1 corresponded to eight isolates from six counties that are 65–380 km apart in São Paulo state. Almost all isolates effected 100% mortality in infected mice and are therefore considered to be virulent. Genotype #2 had eight isolates from four counties 150–500 km apart. The percentage of infected mouse mortality varies from 0 to 100%. Genotype #3 had isolates from two counties that are 150 km apart. None of these isolates killed an infected mouse within 4 weeks p.i. Genotype #4 had four isolates and these isolates were found in two counties 300 km apart. Genotypes #5, #6 and #7 each had two isolates and both isolates were from the same county. Thirteen genotypes (#8 to #20) with single isolates originated from seven counties (Table 1).

Eighteen of the 20 genotypes had allele differences in two to 10 loci, except genotypes #1 and #5 which differed at locus L358 only. Including the CS3 marker in this study did not increase the resolution of typing, even though a new allele (u-1) was identified for TgCatBr41 and TgCatBr76. Several of the multilocus PCR-RFLP markers revealed non-typical Type I, II and III alleles (denoted as u-1 and u-2) even though these markers were originally developed based on DNA sequence polymorphisms among the clonal Type I, II and III lineages. Such unique alleles were identified for markers *SAG1*, *SAG2*, c22-8, *PK1* and CS3.

### 3.2. Analysis of genotypes and virulence in mice

The composite dataset consists of 125 isolates from four states in Brazil. A map of Brazil is presented in Fig. 1. The states and counties where *T. gondii* isolates were obtained are indicated by grey shading and black dots, respectively. These 125 isolates include 44 cat isolates from São Paulo in the current study, 15 recently reported isolates from chickens in Pará, 19 isolates from chickens in Rio Grande do Sul (Dubey et al., 2007b), 28 isolates from cats in Paraná (Dubey et al., 2004; Su et al., 2006) and 19 isolates from dogs in São Paulo city (Dubey et al., 2007a). The summary of genotyping and mortality rate in infected mice is presented in Table 1 and Supplementary Table S1. The PCR-RFLP data was analysed by SplitsTree4 (Huson, 1998; Huson and Bryant, 2006) and the NeighborNet phylogenetic network is presented in Fig. 2.

A total of 48 genotypes were identified from these 125 isolates. Twenty-six of these genotypes had a single isolate. Four of the 22 genotypes with multiple isolates identified from different hosts and locations are considered clonal lineages in Brazil. These lineages are denoted as Types BrI, BrII, BrIII and BrIV (Table 1 and Supplementary Table S1).

Among these four major lineages, Type BrI includes eight cat isolates from São Paulo (see Table 1, Genotype #1), five cat isolates (TgCatBr2, 12, 17, 21, 30) from Paraná, and one chicken isolate (TgCkBr144) from Pará (Supplementary Table S1). The distance between Pará and the states of São Paulo and Paraná is about 3000 km. Interestingly, isolate GPHT from humans in France belongs to this lineage. Infection in mice showed that the majority of Type BrI isolates killed 100% mice infected within 4 weeks, except TgDgBr3 and TgCatBr54. For TgDgBr3, five mice infected with bradyzoites from dog heart tissue died within 2 weeks of infection, whilst three of five mice infected with *T. gondii* from dog brain tissue died within 4 weeks and the other two mice died on days 30 and 53, respectively (Dubey et al., 2007a). By definition, the mortality rate for the TgDgBr3 isolate is 80% (8/10). For TgCatBr54, three of the four mice infected with bradyzoites from mixed tissue preparation died within 4 weeks of infection, and the fourth mouse died on day 30 (Pena et al., 2006). By definition, the mortality rate for the TgCatBr54 isolate is 75% (3/4). Overall, Type BrI isolates are highly virulent in mice. The Type BrII lineage includes eight cat isolates from São Paulo (see Table 1, Genotype #2), two cat isolates (TgCatBr1, 7) from Paraná and two dog isolates (TgDgBr1, 13) from São Paulo city (Supplementary Table S1). The proportion of infected mice killed by Type BrII isolates varies from 0 to 100%, indicating an intermediate virulent phenotype. Type BrIII lineage includes five cat isolates from São Paulo (see Table 1, Genotype #3), two cat isolates (TgCatBr3, 4) from Paraná and two dog isolates (TgDgBr4, 12) from São Paulo city (Supplementary Table S1). Most of these isolates did not kill any infected mice within 4 weeks p.i., except isolate TgDgBr12 which killed 86% (6/7) (Dubey et al., 2007a). A pig isolate from the United States, P89, which was defined as a non-virulent strain (Howe et al., 1996) based on tachyzoite infections, also belong to the Type BrIII

Table 1
Multilocus genotyping of *Toxoplasma gondii* isolates from cats from São Paulo state, Brazil by PCR restriction fragment length polymorphism (RFLP) analysis

| Cats[a] isolate ID | County | % Mortality in mice[a] (No. died/No. infected) | PCR-RFLP genotype | | | | | | | | | | | | Genotypes (virulence) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | SAG1 | 5′+3′ SAG2[b] | SAG2[c] | SAG3 | BTUB | GRA6 | c22-8 | c29-2 | L358 | PK1 | Apico | CS3 | |
| TgCatBr42 | Colina | 100 (5/5) | I | I | I | III | I | II | u-1 | I | I | I | I | I | #1 |
| TgCatBr47 | Conchas | 100 (1/1) | | | | | | | | | | | | | Type BrI |
| TgCatBr53 | E.S. Pinhal | 100 (5/5) | | | | | | | | | | | | | |
| TgCatBr54 | E.S. Pinhal | 75 (3/4) | | | | | | | | | | | | | (Virulent) |
| TgCatBr55 | E.S. Pinhal | 100 (5/5) | | | | | | | | | | | | | |
| TgCatBr62 | Guaíra | 100 (5/5) | | | | | | | | | | | | | |
| TgCatBr71 | Pirassununga | 100 (5/5) | | | | | | | | | | | | | |
| TgCatBr75 | Ribeirão Preto | 100 (1/1) | | | | | | | | | | | | | |
| TgCatBr39 | Araçatuba | 50 (2/4) | I | I | II | III | III | III | I | III | I | II | III | I | #2 |
| TgCatBr51 | E.S. Pinhal | 80 (4/5) | | | | | | | | | | | | | Type BrII |
| TgCatBr52 | E.S. Pinhal | 100 (5/5) | | | | | | | | | | | | | |
| TgCatBr56 | E.S. Pinhal | 100 (4/4) | | | | | | | | | | | | | (Intermediate) |
| TgCatBr61 | Guaíra | 0 (0/1) | | | | | | | | | | | | | |
| TgCatBr68 | Pirassununga | 75 (3/4) | | | | | | | | | | | | | |
| TgCatBr77 | S.J.Rio Preto | 60 (3/5) | | | | | | | | | | | | | |
| TgCatBr78 | S.J.Rio Preto | 100 (5/5) | | | | | | | | | | | | | |
| TgCatBr58 | Guaíra | 0 (0/1) | I | III | III | III | III | III | II | III | III | III | III | III | #3 |
| TgCatBr59 | Guaíra | 0 (0/5) | | | | | | | | | | | | | Type BrIII |
| TgCatBr60 | Guaíra | 0 (0/3) | | | | | | | | | | | | | |
| TgCatBr73 | Ribeirão Preto | 0 (0/5) | | | | | | | | | | | | | (Non-virulent) |
| TgCatBr74 | Ribeirão Preto | 0 (0/5) | | | | | | | | | | | | | |
| TgCatBr44 | Conchas | 60 (3/5) | u-1 | I | II | III | III | III | II | I | I | u-1 | I | II | #4 |
| TgCatBr48 | Conchas | 100 (5/5) | | | | | | | | | | | | | |
| TgCatBr69 | Pirassununga | 75 (3/4) | | | | | | | | | | | | | |
| TgCatBr70 | Pirassununga | 80 (4/5) | | | | | | | | | | | | | |
| TgCatBr45 | Conchas | 67 (2/3) | I | I | I | III | I | II | u-1 | I | III | I | I | I | #5 |
| TgCatBr46 | Conchas | 100 (5/5) | | | | | | | | | | | | | |
| TgCatBr65 | Osasco | 0 (0/5) | IIorIII | III | III | III | III | III | I | III | III | I | III | III | #6 |
| TgCatBr66 | Osasco | 0 (0/4) | | | | | | | | | | | | | |
| TgCatBr79 | S.J.Rio Preto | 100 (1/1) | I | I | I | I | III | I | u-1 | I | I | I | I | I | #7 |
| TgCatBr80 | S.J.Rio Preto | 100 (5/5) | | | | | | | | | | | | | |
| TgCatBr76 | Ribeirão Preto | 100 (5/5) | I | III | III | III | I | III | I | III | III | u-1 | III | u-1 | #8 |
| TgCatBr83 | São Paulo | 0 (0/2) | I | I | I | III | I | III | u-1 | III | III | I | I | I | #9 |
| TgCatBr84 | São Paulo | 100 (5/5) | I | III | III | III | III | III | I | I | I | u-1 | I | II | #10 |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TgCatBr82 | São Paulo | 100 (5/5) | I | I | II | III | III | u-1 | I | I | III | I | II | #11 |
| TgCatBr40 | Araçatuba | 100 (5/5) | I | I | II | I | III | II | I | I | II | I | I | #12 |
| TgCatBr50 | E.S. Pinhal | 0 (0/1) | I | I | I | III | III | II | u-1 | I | III | I | I | #13 |
| TgCatBr57 | E.S. Pinhal | 100 (5/5) | I | I | II | III | III | III | II | I | III | I | I | #14 |
| TgCatBr72 | Pirassununga | 100 (5/5) | I | I | I | III | III | II | u-1 | I | II | I | II | #15 |
| TgCatBr38 | Araçatuba | 40 (2/5) | u-1 | I | u-1 | III | III | III | II | I | III | I | II | #16 |
| TgCatBr41 | Araçatuba | 100 (5/5) | I | III | III | III | I | III | u-1 | I | u-1 | III | u-1 | #17 |
| TgCatBr67 | Pirassununga | 40 (2/5) | I | III | III | III | III | III | I | III | III | III | I | #18 |
| TgCatBr81 | São Paulo | 67 (2/3) | I | III | III | III | III | III | II | III | III | I | I | #19 |
| TgCatBr64 | Marília | 0 (0/5) | I | I | u-1 | III | III | III | u-1 | I | III | I | III | #20 |
| TgCatBr43 | Conchas | 80 (4/5) | I&u-1 | I | II | III | III | III | I&II | I&III | I | II& u-1 | III | I&II | Mixed |
| TgCatBr49 | Conchas | 100 (2/2) | | | | | | | | | | | | | |

u-1 is the new allele that are different from the clonal Type I, II and III alleles.

[a] Based on previous study reported by Pena et al. (2006).

[b] Previous typing was determined at *SAG2* locus by the method of Howe et al. (1997) and reported by Pena et al. (2006).

[c] A new *SAG2* marker based on the 5′ end of the gene sequence (Su et al., 2006).

lineage. Overall, Type BrIII is considered non-virulent in mice. Type BrIV includes seven chicken isolates (TgCkBr147, 148, 151, 154, 160, 162, 163) from Rio Grande do Sul, and MAS, a human isolate from France (Supplementary Table S1). The mortality rate of mice infected with Type BrIV isolates varies, suggesting an intermediate virulence phenotype. It is worth pointing out that the chicken isolate TgCkBr146, the only clonal Type I isolate identified in Brazil, is highly virulent in mice as expected. Furthermore, isolates TgDgBr11, TgCkBr158 and TgCkBr161, belonging to the clonal Type III lineage, did not kill any infected mice and are non-virulent as expected.

Mortality of infected mice was compared between the four alleles (I, II, III and u-1) of marker CS3 (Table 2). Two isolates (TgCatBr43 and 49) with mixed infection were excluded from the analysis. Of the 44 isolates that were inoculated into outbred mice, 84%, 82%, 0% and 100% of mice died within 4 weeks p.i. for the alleles I, II, III and u-1 of the CS3 marker, respectively. The difference is statically significant between alleles I and III ($P < 0.001$) and alleles II and III ($P < 0.001$). No difference was observed between alleles I and II ($P = 0.22$). Due to the small sample size for u-1 allele, it did not provide meaningful information for the statistical test.

Analysing the survival time of all mice infected with the 44 cat isolates during the experimental period (2 months p.i.) showed that mice infected with isolates harbouring allele III at the CS3 locus had a longer survival time compared with those infected with alleles I or II ($P < 0.001$). There was no difference in survival time between infected mice with alleles I and II ($P > 0.05$).

## 4. Discussion

In the present study, genotyping of 44 Brazilian *T. gondii* isolates from cats in São Paulo state by multilocus PCR-RFLP markers revealed 20 genotypes, suggesting a highly diverse parasite population in cats from this region. The magnitude of diversity in *T. gondii* is further appreciated when a composite dataset of 125 isolates from cats, chickens and dogs from four different states of Brazil is analysed. Of the 125 isolates, a total of 48 genotypes were identified. Twenty-six of these genotypes each have a single isolate, 10 genotypes have two isolates each, and 12 genotypes have three or more isolates each. Four of the most prevalent genotypes including Types BrI, BrII, BrIII and BrIV, have 16, 12, nine and seven isolates, respectively (Fig. 2 and Supplementary Table S1), accounting for one-third of the 125 isolates analysed here. More than half of the 48 genotypes have a single isolate which suggests that only a small portion of the parasite population has been surveyed, and more genotypes are expected to be identified in the future. The genetic relationship among the 125 *T. gondii* isolates from Brazil and 15 reference strains from a variety of sources is presented as a NeighborNet phylogenetic network in Fig. 2. Rather than imposing a strictly

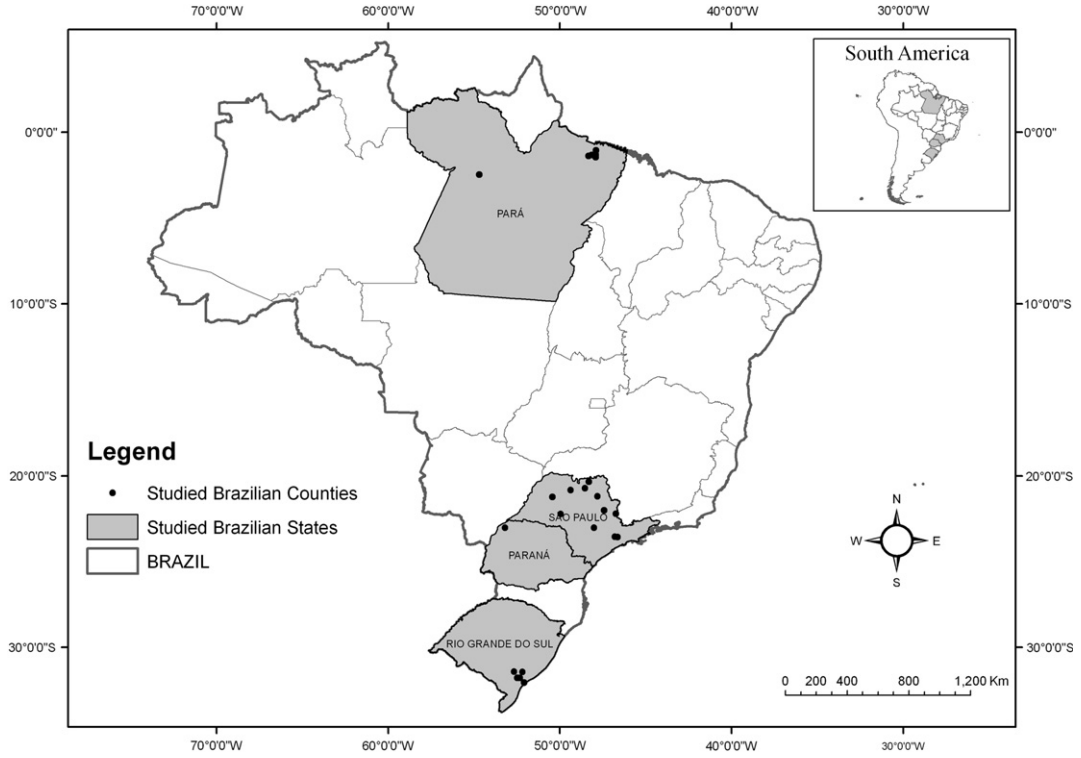


Fig. 1. A map of Brazil. *Toxoplasma gondii* isolates were collected from those states with grey shadings. Black dots indicate the locations (counties) from which those isolates were collected.

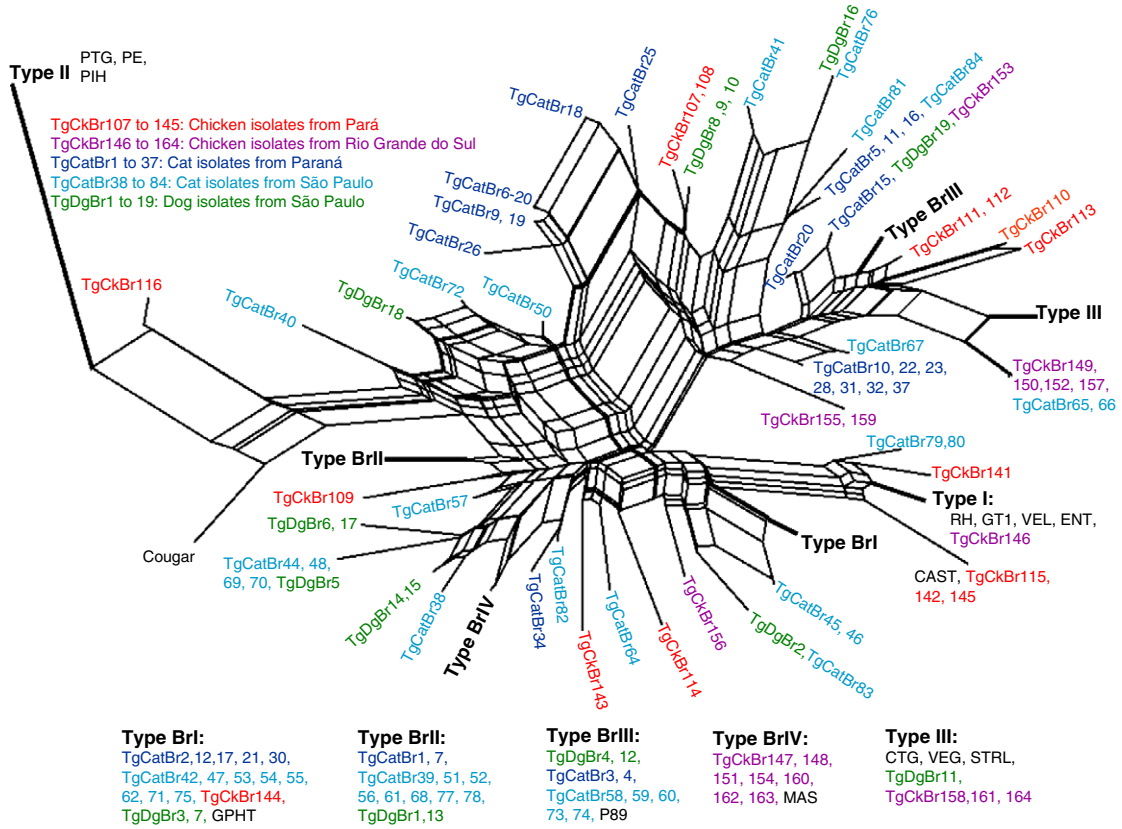


Fig. 2. NeighborNet phylogenetic network of *Toxoplasma gondii* isolates from Brazil. TgCkBr107 to 145: chicken isolates from Pará; TgCkBr146 to 164: chicken isolates from Rio Grande do Sul; TgCatBr1 to 37: cat isolates from Paraná; TgCatBr38 to 84: cat isolates from São Paulo; TgDgBr1 to 19: dog isolates from São Paulo.

Table 2
Mortality rates of mice infected with 44 *Toxoplasma gondii* cat isolates from São Paulo state, Brazil

| | CS3 marker | | | |
|---|---|---|---|---|
| Alleles for CS3 marker | I | II | III | u-1 |
| Number of isolates | 26 | 8 | 8 | 2 |
| Number of mice infected | 99 | 39 | 33 | 10 |
| Number of mice died 4 weeks p.i. (accumulative mortality) | 83 (84%) | 32 (82%) | 0 (0%) | 10 (100%) |

bifurcating structure in a single phylogenetic tree, the phylogenetic network allows a phylogenetic tree with reticulations, which can visually present mutually incompatible trees simultaneously. In a biological context, individuals of a population have complex relationships due to recombination, gene conversion and reassortment, therefore a phylogenetic network is preferred to the traditional bifurcating phylogenetic tree in describing and visually presenting complex relationships in population biology (Morrison, 2005). In a phylogenetic network, the tree-like areas of the diagram represent those parts of phylogeny with no conflict among the data; areas with multifurcations represent those parts of the phylogeny where there is insufficient data to reconstruct the phylogeny; and areas with reticulations represent those parts of the phylogeny where two or more of the phylogenetic patterns conflict with each other (Morrison, 2005). Here, the phylogenetic network of *T. gondii* isolates from Brazil is highly reticulated, suggesting high recombination rates in the parasite population. This finding is in agreement with a previous report that *T. gondii* underwent frequent genetic crosses in Brazil (Lehmann et al., 2004). Clustering of genotypes is evident from the phylogenetic network. The clonal Type I lineage and several genotypes from Brazil are clustered together. The clonal Type III lineage, Type BrIII and several Brazil genotypes are clustered into a major branch. Interestingly, both clonal Type I and III lineages were found in Brazil, however there is not a single Type II isolate identified. Lack of the clonal Type II lineage in Brazil is clearly reflected by the phylogenetic network in Fig. 2. Though more than half (26) of the 48 genotypes identified in Brazil each have a single isolate, several genotypes have multiple isolates, such as Types BrI, BrII, BrIII and BrIV, suggesting that these genotypes have expanded. When comparing genotypes from different animal hosts and geographical locations, no clear clustering of these genotypes is observed. Taken together, our genotyping results strongly suggest that *T. gondii* has an epidemic population structure in Brazil, in which frequent genetic exchange has generated a variety of recombinants and a few successful clonal lineages have expanded into wider geographical areas. This is in sharp contrast to the clonal population structure in North America and Europe, where only three clonal lineages predominate and genetic exchanges among these lineages are rare ( Dardé et al., 1992; Howe and Sibley, 1995; Ajzenberg et al., 2002).

Seropositive rates of *T. gondii* infection in humans and animals are high in Brazil. About 50–80% of adult humans had antibodies to *T. gondii* (Bahia-Oliveira et al., 2003). In several recent studies from which we collected the genotyping data for analysis in this report, high rates of *T. gondii* infection in animals is evident in Brazil. About 35% of 237 cats were seropositive to *T. gondii* in São Paulo state (Pena et al., 2006), whereas 84% of 58 cats were seropositive in cats from Paraná (Dubey et al., 2004). Forty-six per cent of 84 free-range chickens from the states of Rio Grande do Sul and Pará were seropositive (Dubey et al., 2007b). Thirty-six per cent of 118 dogs were seropositive in São Paulo state (Dubey et al., 2007a). The high seropositive rate in cats would predict heavy contamination of the environment by *T. gondii* oocysts, which will lead to contamination of food and water supplies for humans. Indeed, water has been identified as the major source of *T. gondii* transmission to humans in Brazil (Bahia-Oliveira et al., 2003; De Moura et al., 2006). The highly contaminated environment will inevitably lead to a high rate of *T. gondii* infection in intermediate hosts and the consequence is the increased opportunity of genetic recombination in cats. This unique epidemiology of *T. gondii* transmission would lead to and maintain the epidemic population structure of the parasite in Brazil.

*Toxoplasma gondii* virulence in mice depends on several factors, including the stage of the parasite, route, dose, types of mice used and the strain of the parasite (Dubey et al., 2004). It is not possible to control dosage in isolation experiments and tissues from each sample have different doses of the parasite. Dosage has an important role in the outcome of disease and mortality in mice. Therefore, it is difficult to evaluate virulence for any single isolate. However, when multiple isolates (with unknown parasite burdens) from the same genotype were tested in mice, it was expected that different or a range of doses (low and high) of parasites were included. Therefore, if all isolates from the same genotype killed all infected mice, then we can be sure this genotype is mouse-virulent. If none of the isolates from the same genotype killed any infected mice, we can be confident this genotype is not mouse-virulent. Therefore, cumulative mortality of infected mice will be a good indicator of virulence for a given genotype. From a statistical point of view, the more isolates tested for a given genotype, the higher confidence level we can gain on the virulence phenotype. It is particularly reassuring to see that the chicken isolate TgCkBr146 of the clonal Type I lineage is highly virulent in mice as expected. In addition, isolates TgDgBr11, TgCkBr158 and TgCkBr161 of the clonal Type III lineage, did not kill any infected mice and are non-virulent as expected (Supplementary Table S1). Based on the mortality rate in infected mice, it is clear that Type BrI isolates are virulent, Type BrIII isolates are non-virulent, and Types BrII and BrIV isolates are intermediate virulent (Table 1 and Supplementary Table S1). As described above, many isolates from different studies in different Brazilian states, and experimentally inoculated

into mice in different laboratories, that grouped together in the same genotype still have similar virulence phenotype. This indicates that the *T. gondii* biological phenotype in mice is associated with its genotype.

In a previous genetic mapping and linkage analysis study, marker CS3 on chromosome VIIa of *T. gondii* has been shown to be strongly linked to acute virulence in mice (Khan et al., 2005). To determine if it can be used to predict virulence of *T. gondii* isolates, we genotyped the 44 cat isolates from São Paulo state at CS3 locus. The result indicates that isolates with the alleles I and II at the CS3 locus are strongly associated with mortality in infected mice ($P < 0.001$), whilst isolates with the allele III at CS3 is strongly associated with survival of infected mice ($P < 0.001$). This result indicates that the same gene linked to CS3 might be responsible for acute virulence in both the clonal Type I lineage and the atypical lineages in broad geographical regions. Recently, a protein kinase named ROP18, which locates in close proximity to CS3 on chromosome VIIa, was identified to be the virulence gene of Type I lineage of *T. gondii* (Taylor et al., 2006). Therefore, it is likely that ROP18 is involved in the virulence of those isolates collected from Brazil.

It was previously believed that the *T. gondii* population is strictly clonal and the non-clonal isolates (also known as atypical or exotic) identified by RFLP markers were often considered to be recombinants of the clonal Types I, II and III lineages (Ferreira et al., 2006). This view of the "atypical" isolates may not be true. As the parasite population is unique in South America, the RFLP data cannot be directly compared with that of the North American and Europe populations to infer their phylogenetic relationship, due to the effect of population stratification. To address this issue, it is necessary to study *T. gondii* at the DNA sequencing level. Recent study by this approach had confirmed that in Brazil *T. gondii* lineages were highly diverged from the clonal Type I, II and III lineages that predominate in North America and Europe (Khan et al., 2006).

It is of great interest to study the biological differences among different genotypes of *T. gondii* and to investigate whether the genotypes are related to disease manifestations in human toxoplasmosis (Saeij et al., 2005). Previous studies reported that certain strains were more frequently associated with a particular type of toxoplasmosis in human patients (Fuentes et al., 2001; Grigg et al., 2001; Khan et al., 2005, 2006; Vallochi et al., 2005). However, as there is no large scale epidemiological study to reveal the diversity of *T. gondii* genotypes both in human and animal populations, it is not clear if the bias of disease manifestations is due to the background genotypes in the environment where these patients reside, or the consequence of different biological traits that make certain genotypes more virulent in causing a particular type of human disease. Therefore, it is necessary to have a thorough epidemiological study to reveal *T. gondii* population diversity in the environment.

It worth pointing out that genotyping *T. gondii* by the multilocus PCR-RFLP markers has generated invaluable information in revealing the parasite's diversity. The advantage of these multilocus PCR-RFLP markers is that they are easy to use and have a high resolution in identifying *T. gondii* isolates (Su et al., 2006). Even though these markers were originally developed based on DNA sequence polymorphisms in the clonal Type I, II and III lineages, non-clonal alleles (denoted u-1, u-2) are revealed for half of these markers, including SAG1, SAG2 (new), c22-8, c29-2 and PK1. This suggests that many *T. gondii* isolates are highly diverged from the clonal Type I, II and III lineages at the DNA sequence level. It is particularly true when *T. gondii* isolates from a different parasite population such as in Brazil are analysed. Therefore, the multilocus PCR-RFLP markers used in this study may to some extent under-estimate the true diversity of the parasite population. To alleviate this bias, it is necessary to sequence these markers from a broad range of parasite isolates collected worldwide to identify DNA polymorphisms. This sequence information will be useful to select additional restriction endonucleases that recognise non-clonal Type I, II and III alleles, therefore eliminating the bias of these genetic markers.

## Acknowledgements

This work was supported in part by a startup fund to C. Su from the Department of Microbiology, The University of Tennessee, USA, and Research Project no. 2006/53745-0 of Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Brazil, granted to H.F.J. Pena. S.M. Gennari is in receipt of a scholarship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil. We thank Marcos Amaku for statistical analysis and Jucélia de Jesus Pereira for technical assistance.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpara.2007.09.004.

## References

Ajzenberg, D., Banuls, A.L., Tibayrenc, M., Dardé, M.L., 2002. Microsatellite analysis of *Toxoplasma gondii* shows considerable polymorphism structured into two main clonal groups. Int. J. Parasitol. 32, 27–38.

Ajzenberg, D., Banuls, A.L., Su, C., Dumetre, A., Demar, M., Carme, B., Dardé, M.L., 2004. Genetic diversity, clonality and sexuality in *Toxoplasma gondii*. Int. J. Parasitol. 34, 1185–1196.

Bahia-Oliveira, L.M., Jones, J.L., Azevedo-Silva, J., Alves, C.C., Orefice, F., Addiss, D.G., 2003. Highly endemic, waterborne toxoplasmosis in north Rio de Janeiro state, Brazil. Emerg. Infect. Dis. 9, 55–62.

Colombo, F.A., Vidal, J.E., Oliveira, A.C.P., de Hernandes, A.V., Bonasser Filho, F., Nogueira, R.S., Focaccia, R., Pereira-Chiocolla, V.L., 2005. Diagnosis of cerebral toxoplasmosis in AIDS patients in

Brazil: importance of molecular and immunological methods using peripheral blood samples. J. Clin. Microbiol. 43, 5044–5047.

Dardé, M.L., Bouteille, B., Pestre-Alexandre, M., 1992. Isoenzyme analysis of 35 *Toxoplasma gondii* isolates and the biological and epidemiological implications. J. Parasitol. 78, 786–794.

De Moura, L., Bahia-Oliveira, L.M.G., Wada, M.Y., Jones, J.L., Tuboi, S.H., Carmo, E.H., Ramalho, W.M., Camargo, N.J., Trevisan, R., Graça, R.M.T., Silva, A.J., Moura, I., Dubey, J.P., Garret, D.O., 2006. Waterborne toxoplasmosis, Brazil, from field to gene. Emerg. Infect. Dis. 12, 326–329.

Dubey, J.P., Beattie, C.P., 1988. Toxoplasmosis of Animals and Man. CRC Press, Boca Raton.

Dubey, J.P., 2004. Toxoplasmosis – a waterborne zoonosis. Vet. Parasitol. 126, 57–72.

Dubey, J.P., Navarro, I.T., Sreekumar, C., Dahl, E., Freire, R.L., Kawabata, H.H., Vianna, M.C., Kwok, O.C., Shen, S.K., Thulliez, P., Lehmann, T., 2004. *Toxoplasma gondii* infections in cats from Paraná, Brazil: seroprevalence, tissue distribution, and biologic and genetic characterization of isolates. J. Parasitol. 90, 721–726.

Dubey, J.P., Gennari, S.M., Sundar, N., Vianna, M.C., Bandini, L.M., Yai, L.E., Kwok, C.H., Su, C., 2007a. Diverse and atypical genotypes identified in *Toxoplasma gondii* from dogs in São Paulo, Brazil. J. Parasitol. 93, 60–64.

Dubey, J.P., Sundar, N., Gennari, S.M., Minervino, A.H.H., Farias, N.A.d.R., Ruas, J.L., dos Santos, T.R.B., Cavalcante, G.T., Kwok, O.C.H., Su, C., 2007b. Biologic and genetic comparison of *Toxoplasma gondii* isolates in free-range chickens from the northern Pará state and the southern state Rio Grande do Sul, Brazil revealed highly diverse and distinct parasite populations. Vet. Parasitol. 143, 182–188.

Ferreira, A.d.M., Vitor, R.T.G., Melo, M.N., 2006. Genetic analysis of natural recombinant Brazilian *Toxoplasma gondii* strains by multilocus PCR-RFLP. Infect. Genet. Evol. 6, 22–31.

Fuentes, I., Rubio, J.M., Ramírez, C., Alvar, J., 2001. Genotypic characterization of *Toxoplasma gondii* strains associated with human toxoplasmosis in Spain: direct analysis from clinical samples. J. Clin. Microbiol. 39, 1566–1570.

Glasner, P.D., Silveira, C., Kruszon-Moran, D., Martins, M.C., Burnier, M., Silveira, S., Camargo, M.E., Nussenblatt, R.B., Kaslow, R.A., Belfort, R., 1992. An unusually high prevalence of ocular toxoplasmosis in southern Brazil. Am. J. Opthalmol. 114, 136–144.

Grigg, M.E., Ganatra, J., Boothroyd, J.C., Margolis, T.P., 2001. Unusual abundance of atypical strains associated with human ocular toxoplasmosis. J. Infect. Dis. 184, 633–639.

Howe, D.K., Sibley, L.D., 1995. *Toxoplasma gondii* comprises three clonal lineages: correlation of parasite genotype with human disease. J. Infect. Dis. 172, 1561–1566.

Howe, D.K., Summers, B.C., Sibley, L.D., 1996. Acute virulence in mice is associated with markers on chromosome VIII in *Toxoplasma gondii*. Infect. Immun. 64, 5193–5198.

Howe, D.K., Honore, S., Derouin, F., Sibley, L.D., 1997. Determination of genotypes of *Toxoplasma gondii* strains isolated from patients with toxoplasmosis. J. Clin. Microbiol. 35, 1411–1414.

Huson, D.H., 1998. SplitsTree: a program for analyzing and visualizing evolutionary data. Bioinformatics 14, 68–73.

Huson, D.H., Bryant, D., 2006. Application of phylogenetic networks in evolutionary studies. Mol. Biol. Evol. 23, 254–267.

Khan, A., Taylor, S., Su, C., Mackey, A.J., Boyle, J., Cole, R., Glover, D., Tang, K., Paulsen, I.T., Berriman, M., Boothroyd, J.C., Pfefferkorn, E.R., Dubey, J.P., Ajioka, J.W., Roos, D.S., Wootton, J.C., Sibley, L.D., 2005. Composite genome map and recombination parameters derived from three archetypal lineages of *Toxoplasma gondii*. Nuc. Acids Res. 33, 2980–2992.

Khan, A., Jordan, C., Muccioli, C., Vallochi, A.L., Rizzo, L.V., Belfort Jr, R., Vitor, R.W.A., Silveira, C., Sibley, L.D., 2006. Genetic divergence of *Toxoplasma gondii* strains associated with ocular toxoplasmosis, Brazil. Emerg. Infect. Dis. 12, 942–949.

Lehmann, T., Graham, D.H., Dahl, E.R., Bahia-Oliveira, L.M., Gennari, S.M., Dubey, J.P., 2004. Variation in the structure of *Toxoplasma gondii* and the roles of selfing, drift, and epistatic selection in maintaining linkage disequilibria. Infect. Genet. Evol. 4, 107–114.

Lehmann, T., Marcet, P.L., Graham, D.H., Dahl, E.R., Dubey, J.P., 2006. Globalization and the population structure of *Toxoplasma gondii*. Proc. Natl. Aacd. Sci. 103, 11423–11428.

Morrison, D.A., 2005. Networks in phylogenetic analysis: new tools for population biology. Int. J. Parasitol. 35, 567–582.

Pena, H.F.J., Soares, R.M., Amaku, M., Dubey, J.P., Gennari, S.M., 2006. *Toxoplasma gondii* infection in cats from São Paulo state, Brazil: seroprevalence, oocyst shedding, isolation in mice, and biologic and molecular characterization. Res. Vet. Sci. 81, 58–67.

Saeij, J.P.J., Boyle, J.P., Boothroyd, J.C., 2005. Differences among the three major strains of *Toxoplasma gondii* and their specific interactions with the infected host. Trends Parasitol. 21, 476–481.

Su, C., Zhang, X., Dubey, J.P., 2006. Genotyping of *Toxoplasma gondii* by multilocus PCR-RFLP markers: a high resolution and simple method for identification of parasites. Int. J. Parasitol. 36, 841–848.

Taylor, S., Barragan, A., Su, C., Fux, B., Fentress, S.J., Tang, K., Beatty, W.L., Hajj, H.E., Jerome, M., Behnke, M.S., White, M., Wootton, J.C., Sibley, L.D., 2006. A Secreted Serine-Threonine Kinase Determines Virulence in the Eukaryotic Pathogen *Toxoplasma gondii*. Science 314, 1776–1780.

Tenter, A.M., Heckeroth, A.R., Weiss, L.M., 2000. *Toxoplasma gondii*: from animals to humans. Int. J. Parasitol. 30, 1217–1258.

Vallochi, A.L., Muccioli, C., Martins, M.C., Silveira, C., Belfort, J.R., Rizzo, L.V., 2005. The genotype of *Toxoplasma gondii* strains causing ocular toxoplasmosis in humans in Brazil. Am. J. Ophthalmol. 139, 350–351.